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Proprotein convertases are members of a new class of endoproteolytic enzymes that are believed to play important roles in human neoplasia. Based on our previous detection of proprotein convertases in human breast tumors, the present study is designed to study the biological significance of these enzymes in breast cancer. Proprotein convertase gene transfections into MCF-7 human breast cancer cells led to profound changes in the breast cancer cells. MCF-7 cells that over-expressed proprotein convertases have become more dependent on estrogen for growth both *in vitro* and *in vivo* as tumors grown in athymic mice. As well, convertase-transfected breast cancer cells become more resistant to the anti-estrogen Tamoxifen. To further study the role of proprotein convertases in mammary gland development and tumorigenesis, transgenic mice bearing a convertase transgene targeted to the mammary gland have been generated. Characterization of these novel transgenic mice with respect to breast development and tumorigenesis is in progress.

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## INTRODUCTION:

Posttranslational processing is a major mechanism by which the biological activities of many proteins, including protein hormones and cellular growth regulatory proteins are modified. A new class of enzymes that perform protein processing are the Proprotein Convertases. Proprotein convertases, by virtue of their unique property of modifying the biological activities of growth-promoting and growth-suppressing cellular regulatory proteins, are therefore strategically involved in the neoplastic process (reviews in Steiner et al, 92; Mbikay et al, 93; Seidah et al, 98). We have completed a preliminary survey of human breast cancer cell lines as well as 30 primary human breast tumor specimens and 10 normal breast tissue samples; this study has revealed an remarkable increase in the expression of several proprotein convertases (PC1, furin and PACE4, but not PC2) in breast cancer cells (Cheng et al, 97 ). These data suggested that aberrant expression of proprotein convertases may be a hallmark of human breast cancers. A direct linkage between proprotein convertase activity and breast cancer phenotype has been strengthened by the recent finding that the human breast cancer susceptibility gene product and tumor-suppressor, BRCA1, is a member of the granin family of proteins and it may be a natural target of proprotein convertases. **This IDEA proposal seeks to test the hypothesis that elevated expression of proprotein convertases is one mechanism by which breast cancer cells acquire their growth advantage, and that a perturbation (enhancement) of convertase expression in the developing mammary gland of transgenic mice will predispose the mammary gland towards neoplastic transformation.** Two specific objectives will be sought: [1] To determine in human breast cancer cell lines if altered expression of proprotein convertases affect cell growth and posttranslational processing of BRCA1. This will be accomplished by generating a series of MCF-7 human breast cancer cell lines with stable integration of sense or anti-sense convertase cDNAs (PC1 or furin) expression vectors. The growth *in vitro* and *in vivo* in athymic nude of these "transfected" cell lines having over-expression or reduced-expression of convertase will be compared to that of wild type MCF-7 cells. Also, the production of BRCA1 and its processed peptides in "transfected" and wildtype cells will be compared by using BRCA1 specific antibodies. [2] To elucidate the consequences of over-expression of convertases targeted to the mammary glands of transgenic mice in affecting Brca1 (the mouse homolog) processing and cellular transformation. We will target PC1 or furin to the mammary gland using the MMTV promoter. We will monitor for abnormal changes in the mammary glands at various developmental and functional stages. We will measure the profile of Brca1 (the mouse BRCA1 homolog) protein patterns in the same tissues. If over-expression of a convertase alone does not produce discernable abnormality in the transgenic mouse mammary glands, we will produce "double" transgenic mice between MMTV-convertase transgenic mice with MMTV-neu transgenic mice. The "double" transgenic mice will be used to test the hypothesis that convertases facilitate the oncogenic potentials of cellular proto-oncogenes such as neu towards breast tumorigenesis. The parameters to be studied in the "double" transgenic mice will be the same as those proposed for the single MMTV-convertase transgenic mice.

## BODY:

**Tasks 1 to 5: To determine in human breast cancer cell lines if altered expression of proprotein convertases affect breast cancer cell growth.**

We have already completed most of the studies outlined in Tasks 1 to 5. Two pro-protein convertase cDNAs--PC1 and furin--were stably transfected into human breast cancer cell line

MCF-7 to study the altered breast cancer growth behaviour both *in vitro* and *in vivo*. The PC1 and furin over-expressing lines possessed an altered morphology when grown in culture. In the presence of fetal bovine serum, the convertase-over-expressing cells and the wildtype control cells grew with a similar rate. However, when a serum-free defined medium was used, the convertase-transfected cells grew slower than the vector transfected, controlled cells. The cell population doubling times of PC1- and furin-transfected cells were 1.5 times on plastic and 2 times on Matrigel than control cells. When grown *in vivo* in athymic nude mice supplemented with a high dose (5 mg/pellet) of estradiol, there was no significant difference between the growth rates of the convertase-transfected and control MCF-7 tumors. However, when the estradiol dose was reduced to 1.7 mg/pellet, the tumours from PC1 or furin transfected MCF-7 cells grew slower (growth rate 100-200 mm<sup>3</sup>/week) when compared with tumours of controlled MCF-7 (growth rate 585 mm<sup>3</sup>/week). It appears that the over-expression of proprotein convertases has rendered the breast cancer cells more dependent on estrogen for growth. The response to Tamoxifen treatment was also different, with the regression rate (70-130 mm<sup>3</sup>/week) of tumours from PC1 or furin transfected MCF-7 significantly less than their control MCF-7 tumours (547 mm<sup>3</sup>/week). Thus, the convertase-transfected cells have become more anti-estrogen resistant. The results of this study therefore show that an over-expression of proprotein convertases can profoundly influence the growth properties of breast cancer cells *in vitro* and *in vivo*, most notably in their sensitivity to estrogen and anti-estrogen responses. **This work is detailed in a manuscript already submitted for publication (Cheng et al, 1999, appendix).**

**Task 6: To determine in human breast cancer cells if altered expression of proprotein convertases affect the posttranslational processing of BRCA1.**

A series of experiments have been conducted to examine the profiles of proteins and peptide that are immunoreactive to two anti-BRCA1 antibodies. In this study, the consequence of the over-expression of proprotein convertases PC1 and furin on the posttranslational processing of the endogenous BRCA1 protein was assessed by Western immunoblotting of total cellular proteins. The profiles of BRCA-1 immunoreactive peptides in wildtype, PC1-transfected and furin-transfected MCF-7 cells were compared, using two rabbit polyclonal antibodies (C20 is generated against the carboxyl terminal 20 amino acid residues, and D20, the amino terminal 20 amino acids). Our preliminary results are summarized in Figure 1 on page 10. Both C20 and D20 antibodies detected not only the 220K BRCA1 protein but also other minor and major protein species, and this is especially true for D20 (Figure 1, left and middle panels). All the signals disappeared when the antibodies were absorbed with the corresponding 20-residue peptide (data not shown), indicating the signals were specific.

When comparing the profiles of the immunoreactive proteins in proprotein convertase (PC1 and furin) transfected and vector transfected (control) MCF-7 cells, there was no reproducible difference for the 220K BRCA1. However, there appears to be a substantial reduction of a protein doublet of 35-37K, detected by both C20 and D20 antibodies, in both the PC1 and furin transfected MCF-7 cells. At present, the identity of these 35-37K proteins is not known. Since it is known (Wilson et al, 1996) that the C20 antibodies also recognize the ~190K epidermal growth factor receptor (EGFR), we performed a Western blot using anti-EGFR antibodies. Unlike another breast cancer cell line MDA-MB-468 that expresses high levels of EGFR, the MCF-7 cells express low levels of EGFR which was detected by anti-EGFR antibodies (Figure 1, right panel) but not by C20 antibodies (left panel). Importantly, the 35-37K proteins detected by C20, and to a lesser degree by D20 (middle



panel), are not detected by anti-EGFR antibodies, indicating these latter protein doublets are not related to EGFR.

Because the antibodies we have employed (C20 and D20) were generated to 20-mer peptides, they appear not to be able to provide adequate specificity for Western blot analysis. We are repeating the experiments by using a more specific antibody, MS110, which was generated to the first 304 amino acid residues. MS110 has recently been reported to be the antibody of choice in terms of specificity and sensitivity of detection of BRCA1 (Wilson et al, 1999).

**Tasks 7-9: To Construct MMTV-hPC1 and MMTV-hfurin targeting vectors and to generate heterozygous transgenic mice with the targeted expression of human PC1 and human furin in the mammary glands.**

In order to study the role of proprotein convertases in mammary gland development and tumorigenesis, transgenic mice bearing a MMTV-hPC1 or MMTV-hfurin transgene will be studied. We have successfully produced the MMTV-hPC1 targeting vector, and introduced by pronucleus injection into ova of the CD1 mice. The first round of injection, followed by intrauterine replacement, has resulted in 69 live-births. Of these, 9 mice were transgenic for MMTV-hPC1 as determined by Southern hybridization analysis of tail genomic DNA digested with SacI, yielding a diagnostic DNA fragment of 2.9 kb (Figure 2A). Many of the founder mice carry multiple copies of the transgene. Each of the nine founders were bred with wildtype CD1 mice to produce F1 generation of heterozygotic mice. One female F1 mouse from each progeny was tested for transgene expression in the mammary gland, by Northern hybridization. Figure 2B shows that the off-springs of founder mice #3 and #7 expressed the hPC1 transgene that produces a predicted mRNA transcript of approximately 3.9 kb. The two positive transgenic mouse lines (#3 and #7) are currently being bred to homozygosity and then used for future studies on the biological effects of proprotein convertase over-expression on mammary gland development and tumorigenesis. The second round of injection has resulted in the production of one MMTV-PC1 transgenic mouse line (#16) that expresses PC1 mRNA in the mammary gland, bring the total of PC1-expressing transgenic mouse lines to 3.

In the coming year, we will produce new transgenic mouse lines harboring the second convertase transgene, MMTV-hfurin.

**Task 10: Generation of homozygous lines for MMTV-PC1 and MMTV-furin transgenic mice.**

This task has also been completed for MMTV-PC1 transgenic mice. The three MMTV-PC1 transgenic lines described above (#3, #7 & #16) have been successful bred to homozygosity. The production of homozygous MMTV-furin transgenic mice should be completed in the final year of this contract.

**Task 11: Documentation of developmental patterns, proprotein convertase expression and Brca1 protein profiles in transgenic mouse lines.**

This is in progress for MMTV-PC1 lines #3, #7 and #16. We are near completion of collecting mammary glands from the following groups of homozygous MMTV-PC1 transgenic and wild type (non-transgenic) mice: (1) 3-month-old virgins, (2) 7-day pregnant, (3) 18-day pregnant, (4) 10-day lactation, and (5) 10-day post-lactation (involuting). Three animals were used per group. At each developmental and functional stage, we will study histo-morphology, proprotein convertase gene expression, and Brca1 protein profile. The normal pattern of developmental and functional changes in the mouse mammary gland has to be first established and used for comparison to the patterns

observed in transgenic mice. Figure 3 shows the normal histology of wild-type mouse mammary gland in different phases of development (virgin, pregnant) and functions (lactation, involution). We will next establish the normal profiles of the endogenous mouse PC1 and mouse furin gene expression, to be followed by establishing the histological and human PC1 transgene expression profiles in the three homozygous human PC1 transgenic mouse lines.

#### **KEY RESEARCH ACCOMPLISHMENTS:**

- Development of novel human breast cancer cell lines that over-express proprotein convertase genes.
- The discovery that high levels of proprotein convertase expression decreases estrogen sensitivity and increases tamoxifen resistance in human breast cancer.
- Development of novel transgenic mouse models for the investigation of the role of proprotein convertases in breast development and tumorigenesis.

#### **REPORTABLE OUTCOMES:**

- Manuscript submitted for publication:  
Cheng, M., Xu, N., Iwasiow, B., Seidah, N., Chrétien M., and Shiu, R.P.C. (1999) Elevated Expression of Proprotein Convertases Alters Breast Cancer Cell Growth In Response to Estrogen and Tamoxifen
- Abstracts & presentations:  
Cheng, M, Xu, N., Seidah, N., Chrétien M., and Shiu, R.P.C. (1999) Elevated Expression of Proprotein Convertases Alters Breast Cancer Cell Growth In Response to Estrogen and Tamoxifen. Proceedings, 90<sup>th</sup> Annual Meeting, American Association For Cancer Research, abstract #1065, p.160, Philadelphia, PA.
- Development of cell lines:  
Novel MCF-7 human breast cancer cell lines with stable integration of proprotein convertase PC1 and furin transgenes.
- Development of animal models:  
Novel transgenic mouse model harboring a human PC1 convertase transgene targeted to the mammary gland.
- Funding applied for based on work supported by this award:  
An application has been sent to the Medical Research Council of Canada for funding starting October 2000 to support the continuation of the project.

#### **CONCLUSIONS:**

Proprotein convertases are members of a new class of endoproteolytic enzymes that are believed to play important roles in human neoplasia. Based on our previous detection of proprotein convertases in human breast tumors, the present study is designed to study the biological significance of these enzymes in breast cancer. Proprotein convertase gene transfections into MCF-7 human breast cancer cells led to profound changes in the breast cancer cells. MCF-7 cells that over-expressed proprotein convertases have become more dependent on estrogen for growth both *in vitro* and *in vivo* as tumors grown in athymic mice. As well, convertase-transfected breast cancer cells become more resistant to the anti-estrogen Tamoxifen. In addition, preliminary experiments have suggested that the profiles of BRCA1 related peptides may be altered in the convertase-over-expressing breast cancer cells. To further study the role of proprotein convertases

in mammary gland development and tumorigenesis, transgenic mice bearing a convertase transgene targeted to the mammary gland have been generated. Characterization of these novel transgenic mice with respect to breast development and tumorigenesis is in progress.

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#### APPENDICES:

**The appended materials from pages 10 to 39 are unpublished data and should be protected.**

Figure 1: Western blot of MCF-7 cell proteins using anti-BRCA1 antibodies.

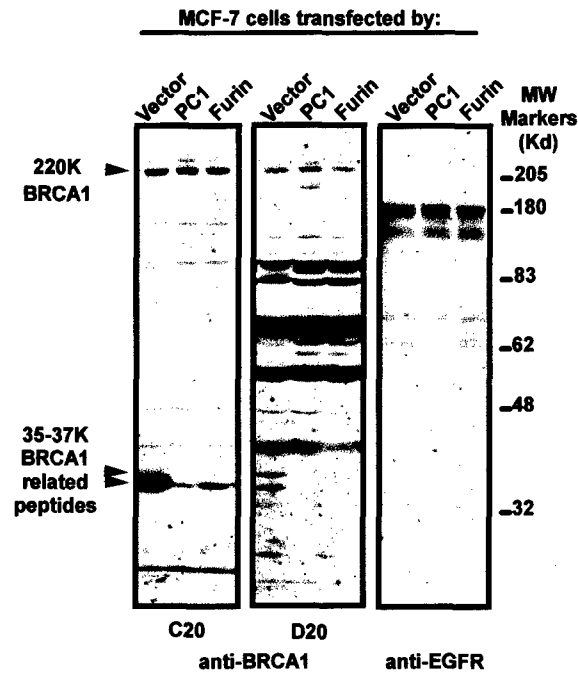
Figure 2: Integration and expression of MMTV-hPC1 transgene in mammary glands of transgenic mice. A. Southern analysis of tail DNA from 13 founder mice; B. Northern analysis of hPC1 transgene expression in mammary gland of F1 off-springs.

Figure 3: Histology of mouse mammary glands in different stages of development and function. A: 8-week-old virgin, B: 20-week-old virgin, C: day-4 pregnant, D: day-10 pregnant, E: day-19 pregnant, F: day-3 lactating, G: day-10 lactating, H: day-3 involuting, I: day-10 involuting.

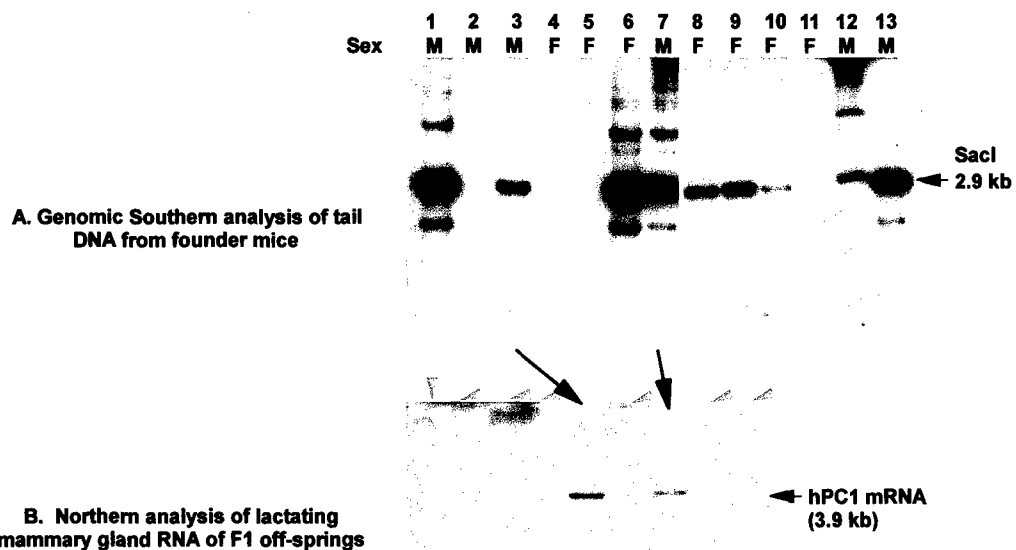
Manuscript appended:

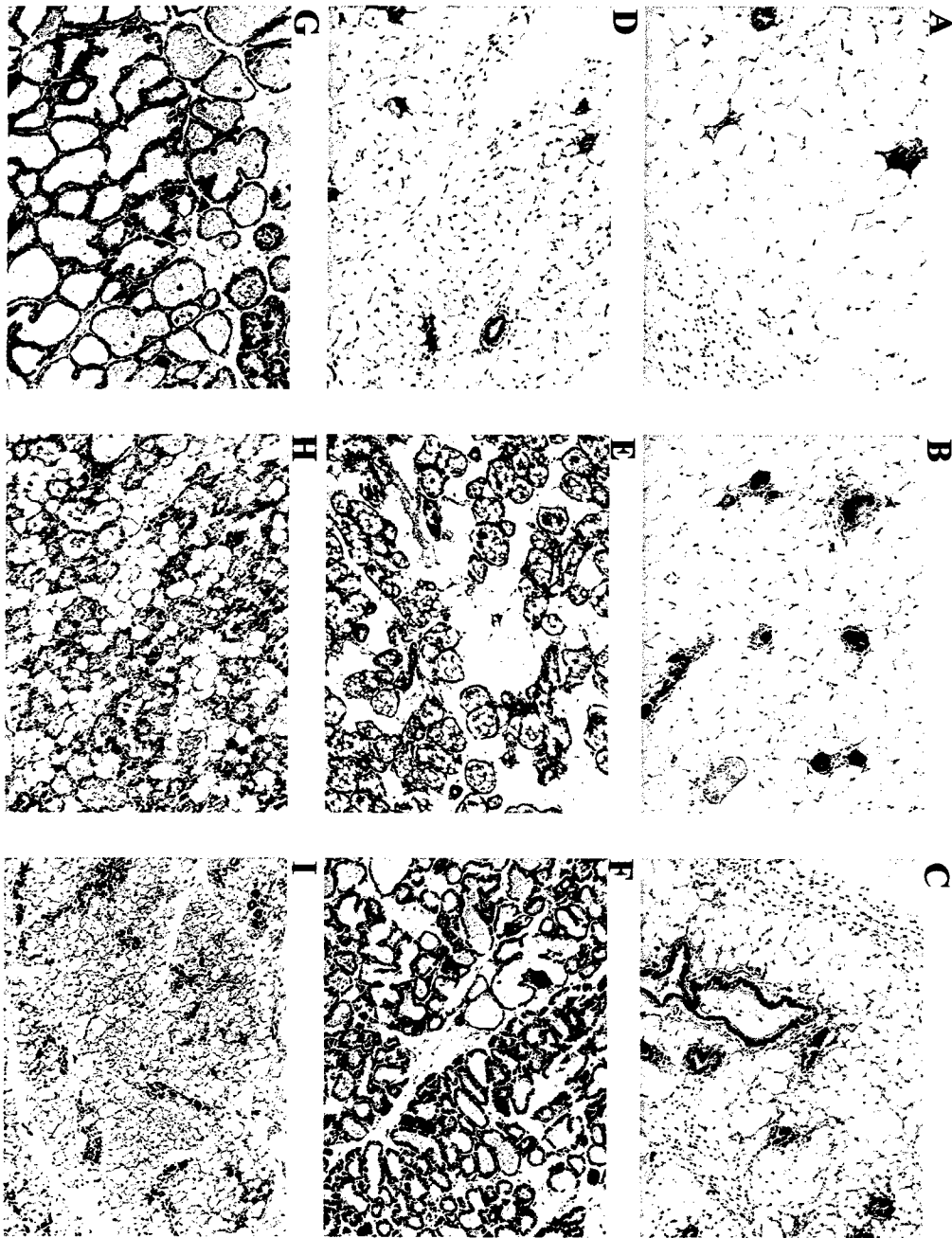
Cheng, M., Xu, N., Iwasiow, B., Seidah, N., Chrétien M., and Shiu, R.P.C. (1999) Elevated Expression of Proprotein Convertases Altered Breast Cancer Cell Growth In Response to Estrogen and Tamoxifen (manuscript submitted for publication)

**Figure 1: Western blot of MCF-7 cell proteins by anti-BRCA1 antibodies**



**Figure 2: Integration of MMTV-hPC1 transgene and expression of hPC1 mRNA in mammary glands of transgenic mice**





**ELEVATED EXPRESSION OF PROPROTEIN CONVERTASES ALTERS BREAST  
CANCER CELL GROWTH IN RESPONSE TO ESTROGEN AND TAMOXIFEN**

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**Running Title:** Proprotein convertases alter breast cancer growth

## SUMMARY

Two proprotein convertase cDNAs, PC1 and furin, were stably transfected into the human breast cancer cell line MCF-7. The PC1 or furin over-expressing cells possessed an altered morphology. When grown *in vitro* in a serum-free medium, the population doubling time of the convertase-transfected cells was twice that of wild-type (WT) cells. High concentrations of estradiol stimulated the growth all three cell types to a similar extent; however, at low concentrations of estradiol, the convertase-transfected cells grew more slowly than WT cells. In athymic nude mice implanted with 5 mg estradiol pellets, the growth of tumours of convertase-transfected MCF-7 cells were stimulated to a similar degree as WT MCF-7 tumours. However, in mice implanted with the lower dose, 1.5 mg estradiol pellets, the tumours of PC1- or furin-transfected MCF-7 cells grew approximately 5 times slower than that of WT MCF-7 cells. In mice implanted with Tamoxifen pellets, tumours of PC1- or furin-transfected MCF-7 cells regressed approximately 5 times slower than the WT tumours. This study shows that the over-expression of proprotein convertases imparts a greater estrogen dependency and anti-estrogen resistance on human breast cancer cells.

**Key words:** breast, cancer, convertase, estrogen, proliferation

## INTRODUCTION

The abnormal expression of autocrine or paracrine growth factors, together with oncogenes, may play a major role in human breast cancer progression (Dickson and Lippman, 1995). Many growth factors (such as epidermal growth factor, transforming growth factors  $\alpha$  and  $\beta$ , insulin-like growth factors IGF I and II) and receptors (such as insulin-like growth factor receptor type I, Neu and integrin), have been shown to be generated from their inactive precursors by the actions of proprotein convertases, or to contain potential processing sites for proprotein convertases (Mbikay et al, 1993). Proprotein convertases, also known as prohormone convertases, are a family of serine proteinases of the subtilisin/kexin type. To date, seven known mammalian convertases have been named: PC1/PC3, PC2, PC4, PC5/PC6, PC7/PC8/LPC/SPC7, furin/PACE and PACE4 (Steiner et al, 1992; Seidah et al, 1998). Six human convertases (except PC4) have been cloned. Thus, an altered expression of proprotein convertases could profoundly influence the growth characteristics of breast and other cancers by changing the production or availability of biologically active growth regulators.

Consistent with this hypothesis is the observation of elevated expression of convertase members in human lung (Schalken et al, 1987; Mbikay et al, 1997) and breast cancers (Scopsi et al, 1995; Cheng et al, 1997). Also, PC7 has been identified at a chromosome translocation breakpoint in a human lymphoma (Meerabux et al, 1996). The over-expression of PACE4 via gene transfection in mouse squamous cell carcinoma resulted in enhanced tumor cell invasiveness (Hubbard et al, 1997). Finally, the observation that the breast cancer susceptibility gene products, BRCA1 and BRCA2, contain numerous potential convertase cleavage sites (Steeg, 1996), is highly suggestive of an important role of proprotein convertases in human breast tumorigenesis. Taken



together, the above findings provide a compelling argument for an important role of the proprotein convertases family of genes in human cancer development and progression.

Recent studies, including our own (Schalken et al, 1987; Mbikay et al, 1997; Scopsi et al, 1995; Cheng et al, 1997), have shown that cancer cells in general possess an elevated expression of proprotein convertases when compared to their normal counterparts. To gain further insight into the biological functions of proprotein convertases in human breast cancer, we have generated via gene transfection MCF-7 human breast cancer cell lines that over-express either one of the two proprotein convertases, PC1 and furin. Here we report that MCF-7 cells over-expressing PC1 or furin possess both an altered cell shape *in vitro* and an altered growth behaviour *in vitro* and *in vivo* in athymic nude mice in response to estrogen and the antiestrogen, Tamoxifen.

## MATERIALS AND METHODS

### Gene transfection of MCF-7 cells.

Full length cDNAs for mouse PC1 (mPC1) and human furin (hfurin) cloned into the expression vector pcDNA3 were linearized with ScaI before being transfected into MCF-7 human breast cancer cells using calcium phosphate (Mammalian Transfection kit and protocol, Stratagene, U.S.A.). MCF-7 cells were also transfected with ScaI-linearized pcDNA3 vector alone and these cells were used as control cells. Cell culture conditions were described as before (Cheng et al, 1997). Neomycin-resistant clones were selected in 1 mg/ml G-418 (Geneticin™) in culture medium. MCF-7 clones were analyzed by Southern blotting for transgene integration, Northern blotting for mRNA expression, and immuno-precipitation or Western blotting for protein production.

### Southern and Northern blot analyses

Genomic DNA was isolated from MCF-7 cell clones as described (Hogan et al, 1986). For Southern analysis, 10  $\mu$ g of DNA was first digested with the appropriate restriction endonucleases: PstI for mPC1-transfected, and BamHI + BglII for hfurin-transfected cells. Hybridization with  $^{32}$ P-labelled mPC1 or hfurin cDNA was carried using conditions as described (Maniatis et al, 1982)

Total RNA was isolated from MCF-7 cells as described (Cheng et al, 1997), and 30  $\mu$ g of each sample was subjected to Northern blot analysis (Maniatis, 1982).

### Immunoprecipitation

MCF-7 cells were labeled with 100  $\mu$ Ci/ml [ $^{35}$ S]-cysteine (ICN, 800 Ci/mmol) for 24 hours in Dulbecco's modified Eagle's medium containing 5% of its normal cysteine. The medium

collected was dialysed and lyophilized. The lyophilized product was dissolved in an immunoprecipitation buffer [IPB, consisting of 50 mM Tris pH 7.5, 100 mM NaCl, sodium deoxycholate 0.5%, SDS 0.1%, NP40 0.5% and 100 K.I.U./ml Trasylol (Aprotinin)]. In order to immunoprecipitate the [ $^{35}$ S]-labeled mPC1 protein from the medium, a rabbit anti-PC1 serum (Basak et al, 1995) directed against the C-terminal peptide sequence was used; normal rabbit serum was used as control. The detailed procedures for immunoprecipitation, analysis by SDS polyacrylamide gel electrophoresis, and finally visualization by fluorography of immunoprecipitated proteins, have been described elsewhere (Shiu and Iwasiow, 1985).

#### **Western blot analysis**

Cultured cells were scraped, pelleted by centrifugation, and lysed in 50 mM Tris-HCL buffer containing 20 mM EDTA, 5% SDS, 5 mM  $\beta$ -glycerophosphate, 1 mM AEBSF [4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride] and 70 K.I.U./ml aprotinin. Protein concentrations were determined by the Bio-Rad (Hercules, CA) protein assay kit. Fifty micrograms of each cell lysate was separated on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose (Bio-Rad). To detect hfurin protein, a polyclonal rabbit anti-furin antiserum (Basak et al, 1995) was used as the first antibody. The second antibody used was horseradish peroxidase conjugated goat anti-rabbit IgG. Visualization was accomplished using the Supersignal<sup>®</sup> detection system (Pierce, USA) according to the manufacturer's instructions.

#### **Cell growth *in vitro***

**Electronic Cell Counting:** Cells were plated in 24-well plates at  $1.25 \times 10^4$  cells/well in a medium containing 5% FBS and allowed to attach overnight. The culture medium was then changed to serum-free medium supplemented with human transferrin (10  $\mu$ g/ml) and bovine serum

albumin (200  $\mu\text{g/ml}$ ) (Karey and Sirbasku, 1988). Two types of cell culture surfaces were used: plastic and plastic coated with the basement membrane Matrigel™ matrix (Becton Dickinson Labware, U.S.A.) according to the manufacturer's protocol. After the prescribed culture period (up to 8 days), the cells were detached with trypsin (for cells on the plastic surface) or dispase (for cells on Matrigel™ matrix) and counted by a Coulter particle counter (Model ZBi, Coulter Electronics, Hialeah, FL). For each determination, the average cell number of 4 wells was used. The cell doubling time was defined as the time (days) required for the cell number to double during the logarithmic growth phase.

**Crystal Violet staining assay:** Cells were plated in 96-well microtiter plates at three thousand cells per well and allowed to attach overnight. The culture medium was then changed to a serum-free medium that was supplemented with human transferrin and bovine serum albumin, as above, as well as different concentrations of estradiol-17 $\beta$ . At each time point, the Crystal Violet staining assay (Alfa and Jay, 1988), based on the binding of the Crystal Violet dye to total cellular proteins, was used. The cell-bound dye was extracted with methanol and absorbance at 590 nm was determined using an ELISA plate reader (Bio-Tek Instruments, Inc., Burlington, VT, U.S.A.)

#### **Cell growth *in vivo***

Six- to seven-week old female Balb/c or CD-1 strain athymic nude mice, obtained from Charles River Canada, St. Constant, Québec, were kept inside a laminar-flow air filtration system. Two doses of 90-day release estradiol pellets were used: 5 mg/pellet (to achieve a blood level of >900 pg/ml) and the 1.7 mg/pellet (to achieve a blood level of 500-600 pg/ml). Placebo pellets were used in control animals. The estradiol, placebo and Tamoxifen (see later) pellets were

obtained from Innovative Research of America, Sarasota, Florida. Each pellet was implanted subcutaneously in the dorsal midline, caudal to the neck, through a small incision which was sealed by Vetbond™ tissue adhesive (3M Animal Care Products, St. Paul, MN).

Breast cancer cells suspended at  $2-5 \times 10^6$  cells/50  $\mu$ l in culture medium were injected subcutaneously into the flanks of the animals, one cell type on each flank. Each experimental group had four mice. For each experimental group, there was a correspondent group with placebo pellet implanted. Tumor volumes were monitored every 7 days by caliper measurement of the 3 dimensions (a,b,c) and were calculated using the formula for a ellipsoid ( $v=4/3\pi abc/2$ ). After the final tumour measurement at the 6th week, the estrogen pellets were removed and Tamoxifen pellets (5.0 mg/pellet, to achieve a blood level of 2-2.5 ng/ml) were implanted. The tumour volumes for the subsequent 5 weeks were measured and were expressed as percentages of the tumour volume achieved prior to the removal of the estradiol pellets.

#### **Estrogen receptor assay**

Two days prior to the harvesting of cultured cells for estrogen receptor determination, the growth medium was changed to one that contained 5% charcoal-stripped fetal bovine serum in order to minimize receptor occupancy due to the presence of estrogens in fetal bovine serum. Estrogen receptors were then determined in cell lysates by the Breast Steroid Receptor Laboratory, University of Manitoba, using the standard ligand-binding assay (McGuire, 1973).

#### **Statistical analysis**

One-way analysis of variance was used to analyze the estrogen receptor contents of different cell types. Linear regression analysis was performed to analyze cell growth *in vitro* and *in vivo*. Statistics analyses were performed using SigmaStat (SPSS Inc., Chicago, IL, U.S.A.).

## RESULTS

### Transfection and expression of proprotein convertases mPC1 and hfurin in MCF-7 human breast cancer cells

MCF-7 cell clones surviving G-418 selection were first analyzed by Southern hybridization of genomic DNA for the integration of an intact transfected proprotein convertase cDNA (mPC1 or hfurin). Fig. 1A-I and 1A-II show the hybridization of a  $^{32}\text{P}$ -labelled mPC1 cDNA probe to the expected 2.0 and 0.4 Kb genomic DNA fragments in a clone positive for mPC1 cDNA integration (designated as clone PC1). A G-418 resistant clone that was transfected with the vector pcDNA3, designated as wild type (clone WT) MCF-7, was included for comparison. Clone PC1 also expressed high levels of mPC1 mRNA (Fig. 1A-III) when compared with the control Clone WT. The level of the endogenous human PC1 (hPC1) mRNA in clone WT was low relative to that of the transfected mPC1 mRNA in clone PC1, and was not normally detectable when total RNA was analyzed by Northern hybridization. However, the endogenous hPC1 mRNA was readily detected in MCF-7 cells by the reverse transcription-polymerase chain reaction (RT-PCR) protocol, as reported by us previously (Cheng et al, 1997).  $^{35}\text{S}$ -cysteine labelled medium of Clone PC1 were subjected to immuno-precipitation using anti-PC1 antibodies. The predominant protein detected was an approximately 85 kDa mPC1 species specifically recognized by the antibodies (Fig. 1A-IV).

MCF-7 cells transfected with hfurin cDNA were similarly analyzed for hfurin integration and expression.  $^{32}\text{P}$ -hfurin cDNA was shown to hybridize to an expected 1.4 Kb genomic DNA fragment in a G-418 resistant clone, designated as clone Furin (Fig. 1B-I and 1B-II). Clone Furin also expressed the expected 3.0 Kb hfurin mRNA (Fig. 1B-III). The endogenous furin transcript,

similar to the endogenous hPC1 mRNA, was low in abundance and was only detected by RT-PCR (Cheng et al, 1997) but not readily by Northern analysis of total RNA samples. When total cellular proteins were subjected to Western blot analysis, a prominent, approximately 90 kDa furin protein was detected by the anti-hfurin antibodies in Clone Furin (Fig. 1B-IV).

### **Morphology of mPC1 and hfurin over-expressing MCF-7 cells**

The WT MCF-7 cells growing on plastic culture dishes showed typical epithelial-like features - flat and polygonal. PC1- or furin-transfected cells, however, demonstrated an altered morphology, in that they were more refractile and spindly, and possessed more prominent cellular processes (Fig. 2). On Matrigel™ coated substratum, however, there was no obvious morphological difference among these three kinds of cells (Fig. 2). All three cell types grew in clusters which expanded in size as cell proliferation proceeded.

### **Effect of over-expression of proprotein convertases on MCF-7 cell proliferation *in vitro***

The proliferation of the PC1 and furin over-expressing MCF-7 clones (designated clone PC1 and Furin, respectively) was first compared to that of the vector-transfected, wild type control MCF-7 cells (clone WT) on plastic substratum and on an extracellular matrix (Matrigel™) substratum, in the presence or absence of fetal bovine serum. Table 1 shows that there was no significant difference in the growth rates of clones PC1, Furin and WT when grown in the presence of 5% fetal bovine serum. When a serum-free medium supplemented with transferrin and bovine serum albumin was used, the doubling time of convertase-transfected clones, PC1 and Furin, was 1.5 times that of WT cells grown on plastic, and 2.0 times that of WT cells grown on Matrigel™. Thus, the over-expression of PC1 or furin significantly reduced the growth rate of the human breast cancer cells MCF-7 *in vitro* in serum-free medium.

Since estrogenic steroids are the major growth-stimulating factors normally present in fetal bovine serum and are absent in serum-free medium, we next studied the effects of estradiol-17 $\beta$  supplementation to serum-free medium on the proliferation of wild-type and convertase-transfected MCF-7 cells on plastic substratum. The doubling times (dt) of the three cell types in the absence and presence of different concentrations of estradiol were recorded (Fig. 3). In the absence of estradiol, clone WT (dt=2.2 days) grew twice as fast as clones PC1 and Furin (dt=4.3 days). All three cell types responded to the addition of estradiol. However, at low concentrations of estradiol ( $< 10^{-12}$  M), the convertase-transfected clones (dt=2.6-3.2 days) still grew more slowly than WT (dt=1.8 days). At higher concentrations of estradiol ( $> 10^{-10}$  M), the growth of PC1 and Furin was further accelerated to a rate (dt=1.8-2.2) similar to that achieved by WT (dt=1.8). Thus, convertase-transfected cells exhibited retarded growth *in vitro* only in the absence, or at low concentrations, of estradiol. High concentrations of estradiol were able to restore the maximal growth potential of convertase over-expressing cells. Further, this higher dependency on estrogen for growth of the convertase-transfected cells appeared not to be associated with a significant alteration in the levels of functional estrogen receptors in these clones; the estrogen receptor contents for WT, PC1 and Furin clones were  $34 \pm 16$ ,  $72 \pm 18$  and  $87 \pm 47$  femtomoles per mg protein, respectively (the differences between these values were not statistically significant,  $p > 0.1$ ).

#### **Growth of PC1- and furin-transfected MCF-7 cells *in vivo* in athymic nude mice**

Estradiol-17 $\beta$  is essential for MCF-7 cell growth *in vivo* in athymic nude mice (Shafie and Grantham, 1981). In the first experiment, 5.0 mg estradiol pellets (blood level  $> 900$  pg/ml), were implanted subcutaneously into the nude mice. Under the influence of this high concentration



of estradiol, there was no significant difference ( $p > 0.2$ ) between the growth rate of WT tumours ( $867 \pm 304 \text{ mm}^3/\text{weeks}$ ) and PC1-transfected cells ( $458 \pm 114 \text{ mm}^3/\text{week}$ ) (Fig. 4A). When lower dose (1.7 mg) estradiol pellets (blood level 500 pg/ml) were used, the WT MCF-7 tumours grew at a rate ( $585 \pm 150 \text{ mm}^3/\text{week}$ ) similar to that in high dose estradiol, but the PC1-transfected MCF-7 tumours grew more slowly ( $93 \pm 14 \text{ mm}^3/\text{week}$ ) (Fig. 4B). Consequently there was a significant 5-fold reduction ( $P < 0.03$ ) in the growth rate of PC1 over-expressing MCF-7 tumours. In a third experiment, the growth of clone PC1 was compared to that of clone Furin in nude mice with the low dose estradiol pellets. As shown in Fig. 4C, there was no difference ( $p > 0.5$ ) between the two MCF-7 clones. Therefore, both PC1- and furin-transfected cells grew more slowly than WT cells *in vivo* in nude mice in the presence of the lower dose estradiol. All three clones failed to grow in the athymic mice implanted with placebo pellets (not shown).

The sensitivity of the established tumours to the anti-estrogen, Tamoxifen, was also evaluated. After 6 weeks of growth, the estradiol pellets were removed and Tamoxifen pellets (5.0 mg/pellet) (blood level 2-2.5 ng/ml) were implanted. The WT MCF-7 tumours regressed four times faster than PC1- or furin-transfected tumours (Fig. 5A and 5B); the WT tumours decreased with a rate of  $547 \text{ mm}^3/\text{week}$ , while PC1- and furin-transfected tumours regressed at the rates of  $71\text{-}113 \text{ mm}^3/\text{week}$  and  $134 \text{ mm}^3/\text{week}$ , respectively ( $p < 0.01$ ).

## DISCUSSION

We have previously demonstrated by using a sensitive method of reverse transcription-polymerase chain reaction (RT-PCR) that human breast cancers, when compared to normal breast tissues, exhibit an elevated mRNA levels for at least four members of the proprotein convertase family-PC1, PC7, furin, and PACE4 (Cheng et al, 1997). In the present study, we have studied some of the biological consequences of over-expression of PC1 and of furin in the human breast cancer cells MCF-7. PC1 was chosen as representative of proprotein convertases that are localized to secretory granules and are responsible for the cleavage of proteins secreted by the regulated secretory pathway. Furin was chosen to represent a membrane-anchored convertase enzyme that is localized to the trans-Golgi network and plasma membrane, and cleaves proteins as they pass through the trans-Golgi route to be secreted by the constitutive secretory pathway (Molloy et al, 1994).

The first noticeable phenotypic change in the MCF-7 clones that over-expressed PC1 or furin was that of an altered cell shape. The transfected MCF-7 cells have a refractile, elongated and spindle morphology, with elongated cell processes (Fig. 2) when grown on plastic substratum. The mechanisms responsible for this altered morphology are not known; however, it is possible that the excess production of PC1 and furin may have altered the processing, and therefore functions, of important cell adhesion proteins that may include integrins, laminin, fibronectin and collagens. Indeed, furin has been shown to cleave integrin pro-alpha 3 and 6 subunits and is believed to be involved in the endoproteolytic processing of integrins (Lehmann et al, 1996). Thus, an altered cell adhesion and morphology seen with our convertase-transfected cells on plastic substratum may be a consequence of changes in integrin function. However, it appears that

exogenously supplemented extracellular matrix proteins could compensate for the loss of function of endogenous adhesion molecules.

In addition to morphological changes, the most striking effect of convertase over-expression in the MCF-7 breast cancer cells was on their sensitivity to hormones affecting cell proliferation. In experiments *in vitro* and *in vivo* (implanted in athymic nude mice), the growth of convertase-transfected cells was less sensitive to estradiol than that of wild-type cells. While the wild-type MCF-7 tumours grew quite efficiently in the presence of low levels of estradiol, the convertase over-expressing tumours required higher levels of estradiol for maximal growth. Thus, it appears that the over-expression of proprotein convertases has rendered the breast cancer tumours more estrogen-dependent.

The *in vivo* study also revealed that the convertase-transfected MCF-7 tumours, once established in the presence of estradiol supplement, regressed at a rate that was 1/5th that of wild type MCF-7 tumors in the presence of the antiestrogen Tamoxifen (Fig. 5). Thus, the over-expression of convertase has also rendered the breast cancer cells more Tamoxifen-resistant.

It is presently unclear as to the mechanism by which the over-expression of proprotein convertases leads to reduced estrogen sensitivity and increased Tamoxifen resistance in MCF-7 cells. It was not due to an alteration of estrogen receptor levels because the estrogen receptor contents were not quantitatively different between the wild-type and convertase-transfected MCF-7 cells. Since both the estrogen-induced cell proliferation and Tamoxifen-induced tumor regression involve estrogen receptor-mediated induction of gene and cellular functions, it appears likely that the over-expression of convertases has affected the activities of co-activators or co-repressors of estrogen receptor function (McKenna et al, 1999). Alternatively, the over-expression of

convertases may impact on signal transduction pathways that "cross-talk" with the estrogen receptor (Smith, 1998), leading to an alteration of estrogen receptor functions. For example, a high level of convertase activity may produce those aberrations in receptor phosphorylation that diminish the affinity of the estrogen receptor for its ligands, estradiol and Tamoxifen; such a change would result in a lower sensitivity of the convertase over-expressing cells to both the receptor agonist and antagonist. Finally, it is also possible that the over-expression of proprotein convertases diminishes the activities of signal molecules downstream to the estrogen receptor. This effect would also produce a breast cancer cell population that is less sensitive to both estradiol and Tamoxifen.

In conclusion, the present study shows that the over-expression of proprotein convertases can profoundly influence the growth behaviour of human breast cancer cells, notably in their responsiveness to estrogen and anti-estrogen actions. Clinically, proprotein convertases may be potentially useful indicators of breast cancers with high estrogen dependency and anti-estrogen resistance.

### ACKNOWLEDGEMENTS

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### LEGENDS TO FIGURES

**Fig. 1A Detection of mPC1 integration and expression in transfected MCF-7 cells.** I. PstI restriction map of integrated mPC1 cDNA. II. Southern analysis of PstI digested genomic DNA revealed the expected 2 Kb and 0.4 Kb bands were present in clone PC1 but not in WT. III. Northern analysis revealed a 2.5 Kb mPC1 transcript in clone PC1 but not in clone WT. <sup>32</sup>P-labelled mouse PC1 cDNA was used as a probe for both Southern and Northern analyses. IV. Immunoprecipitation was carried out by incubating <sup>35</sup>S-cysteine labelled proteins derived from the conditioned medium of clone PC1 with either rabbit anti-PC1 antiserum (AS) or normal rabbit serum (NS) as control. A 85 kDa band was precipitated with anti-PC1 antiserum.

**Fig. 1 B Detection of hfurin integration and expression in transfected MCF-7 cells.** I. Restriction enzyme map of the integrated hfurin cDNA. II. Southern analysis of genomic DNA digested with BamHI and BglII, and using <sup>32</sup>P-labelled hfurin cDNA as a probe. The presence of the transfected hfurin cDNA generated the expected 1.4 Kb band in clone Furin. The endogenous hfurin gene has yielded several additional bands common to both the Furin and WT clones. III. Northern analysis revealed the expected 3.0 Kb transcript in clone Furin. IV. Western blot analysis revealed a 90 kDa protein detected by anti-hfurin antibodies in the clone Furin cells.

**Fig. 2 Morphology of mPC1- and hfurin-transfected MCF-7.** Phase-contrast photomicrographs showing the appearance of clones PC1, Furin and WT cells grown on plastic and Matrigel™.

**Fig. 3 Effect of estradiol-17β on the proliferation of convertase over-expressing and WT MCF-7 *in vitro* on plastic.** Population doubling times (days) were computed from the logarithmic

growth phase of cells under the influence of different concentrations of estradiol. Each value represents the mean  $\pm$  SEM of three independent experiments. Some of the SEM bars for WT are not visible because they are masked by the symbol.

**Fig. 4 Proliferation of mPC1- and hfurin-transfected and wild type MCF-7 cells in athymic nude mice receiving 5 mg (A) and 1.7 mg (B, C) estradiol pellets.** For experiments A and B, two millions of clone PC1 and WT cells were injected subcutaneously in the opposite flanks of balb/c athymic nude mice. For experiment C, 5 millions of clone PC1 and clone Furin cells were injected subcutaneously in the opposite flanks of CD1 athymic nude mice. Each point represents the mean of quadruplicate tumour volumes ( $\text{mm}^3 \pm \text{SEM}$ ). The tumour growth rates ( $\text{mm}^3/\text{week}$ ) were included in the panels. A. The growth rates of WT and PC1 under the influence of 5 mg estradiol pellets were not significantly different ( $p > 0.2$ ). B. The growth rates of WT and PC1 under the influence of 1.7 mg estradiol pellets were significantly different ( $p < 0.03$ ). C. The growth rates of clones PC1 and Furin under the influence of 1.7 mg estradiol pellets were not significantly different ( $p > 0.5$ ).

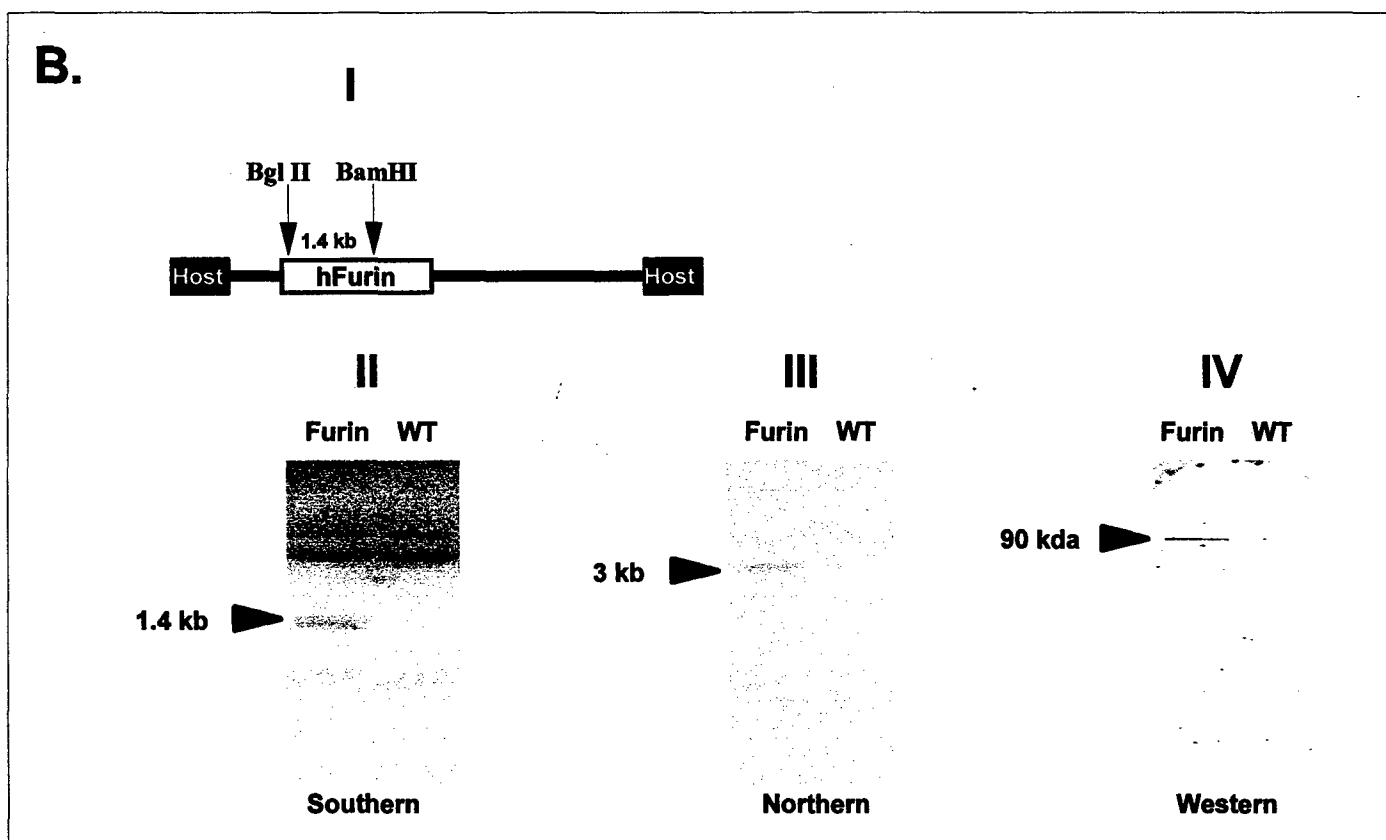
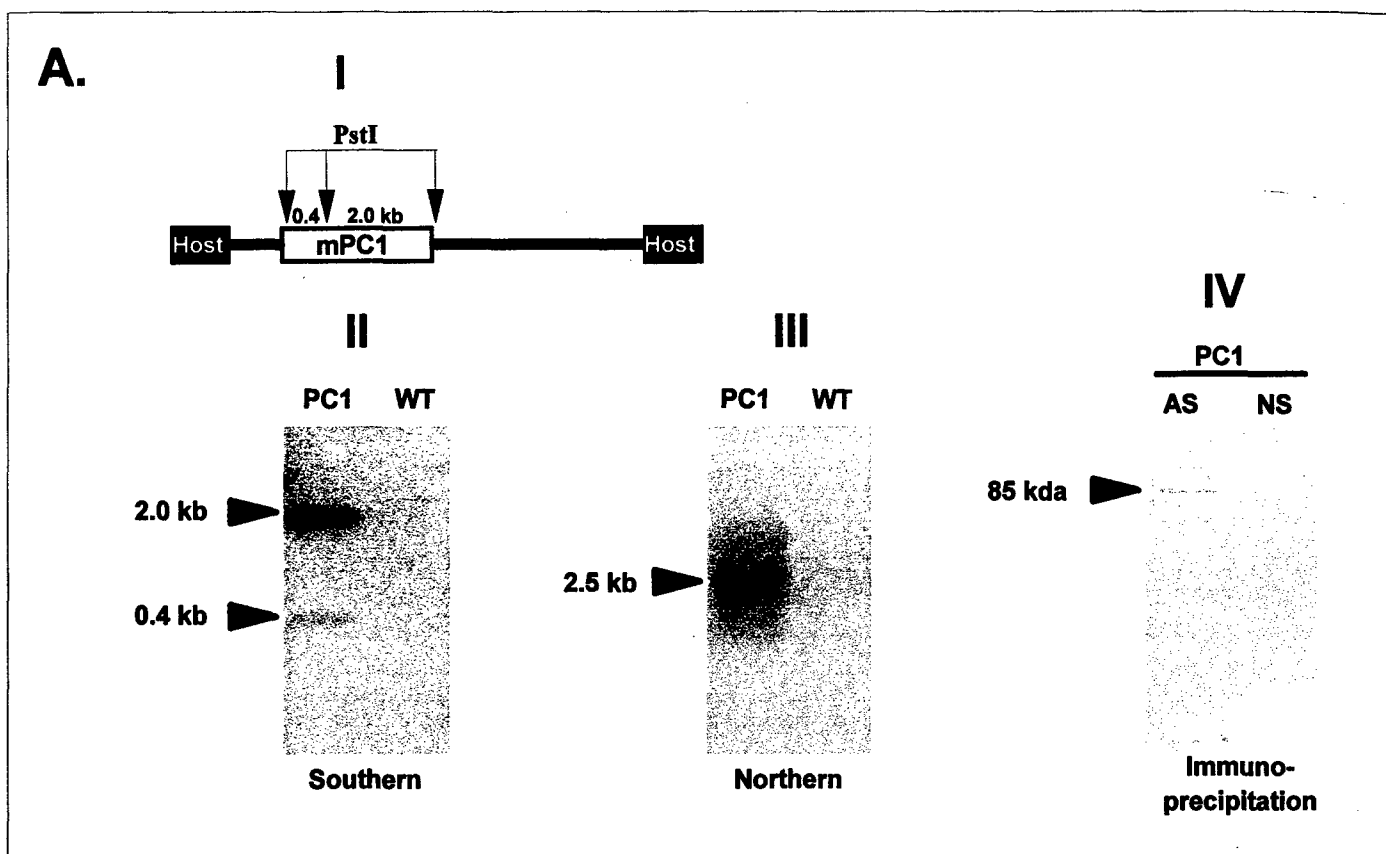
**Fig. 5 Tamoxifen-induced regression of convertase-transfected and wild-type MCF-7 tumours in athymic nude mice.** Tumours were allowed to grow in the presence of estradiol pellets for 6 weeks. The estradiol pellets were then removed, Tamoxifen pellets (5 mg/pellet) were implanted. The day of estradiol pellet removal was taken as week 0, and the tumour volume taken was considered 100%. Each point represents the mean volume ( $\pm$  SEM) of four tumours. A. The regression rate of PC1 tumours was significantly different from that of WT tumours ( $p < 0.02$ ). B. The regression rate of PC1 tumours was not significantly different from that of Furin tumours ( $p > 0.4$ ).

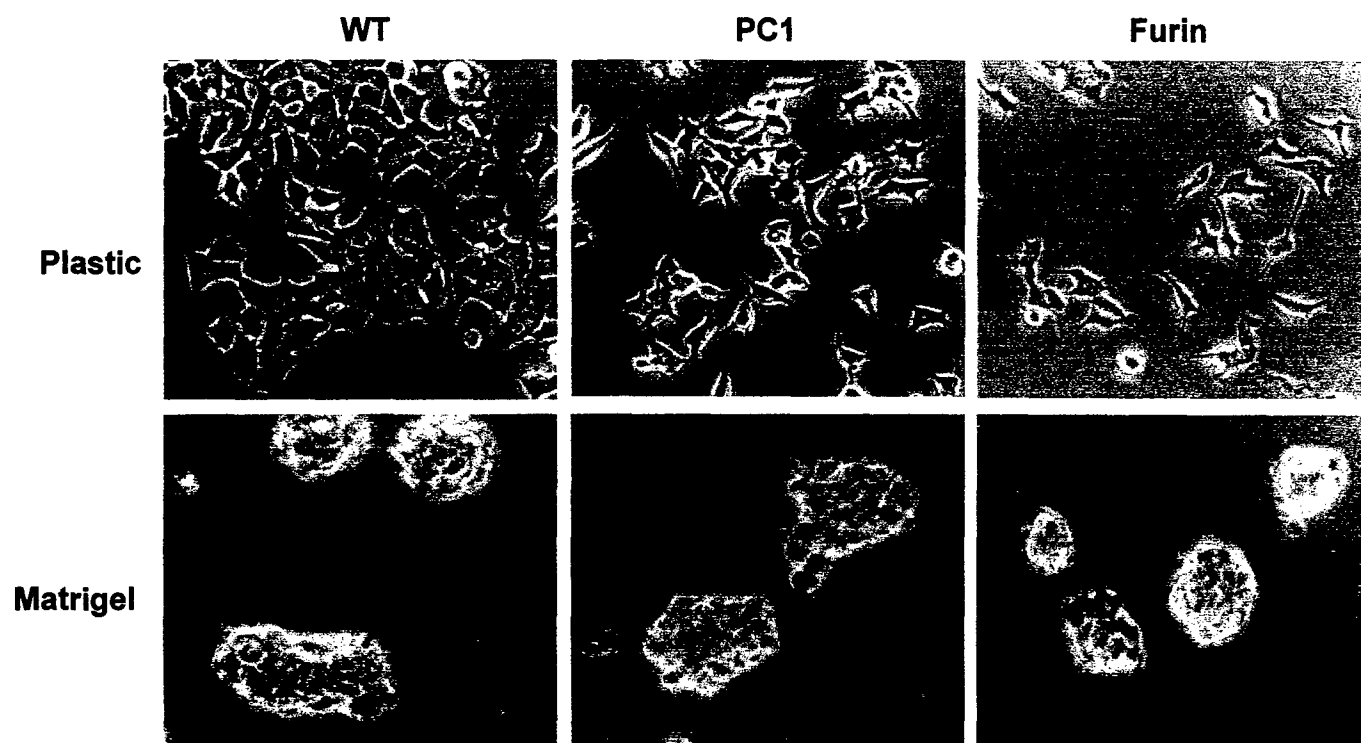
Table 1. Comparison of Cell Growth (doubling time in days) *in vitro*.

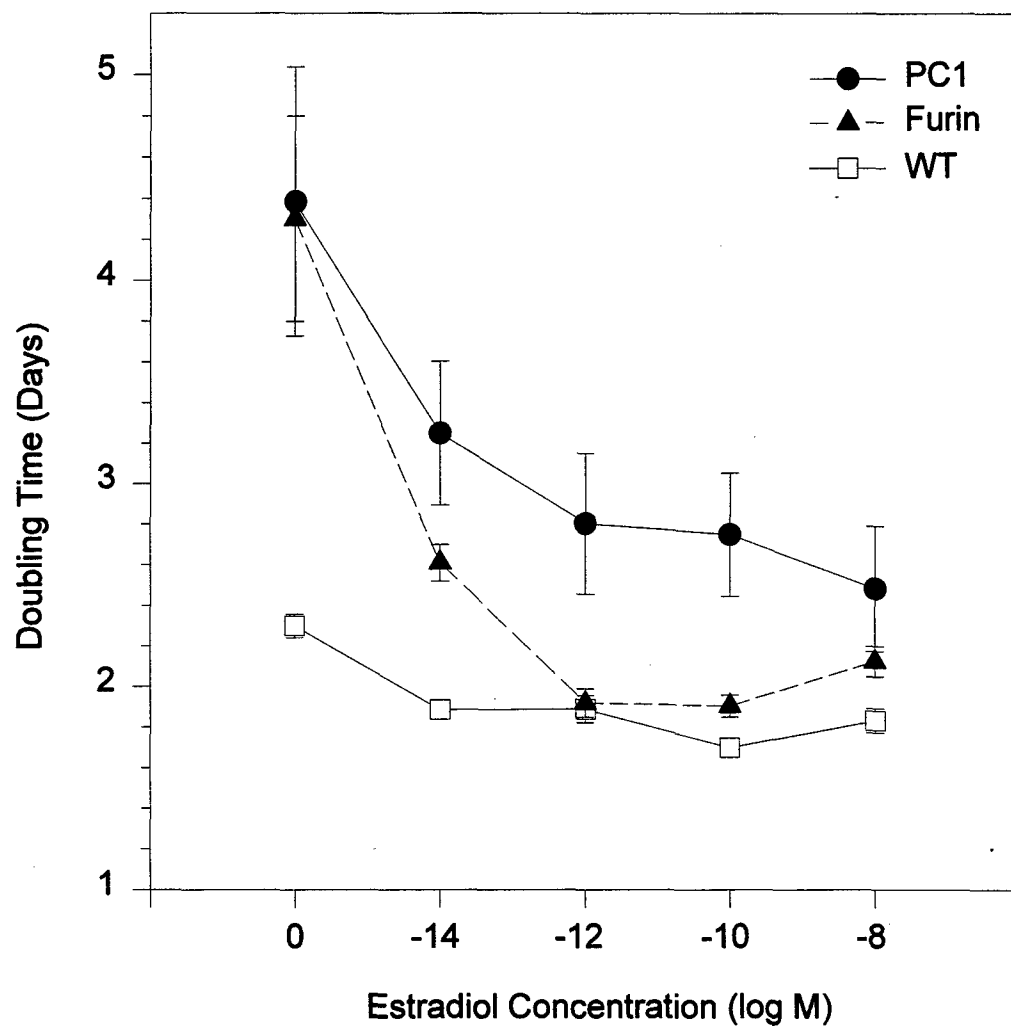
Cell Type	5% FBS	Serum-free	
	Plastic	Plastic	Matrigel™
WT	1.9 ± 0.03	2.58 ± 0.31	1.77 ± 0.03
PC1	2.1 ± 0.12	3.87 ± 0.22*	3.18 ± 0.26*
Furin	2.3 ± 0.21	4.18 ± 0.37*	3.83 ± 0.73*

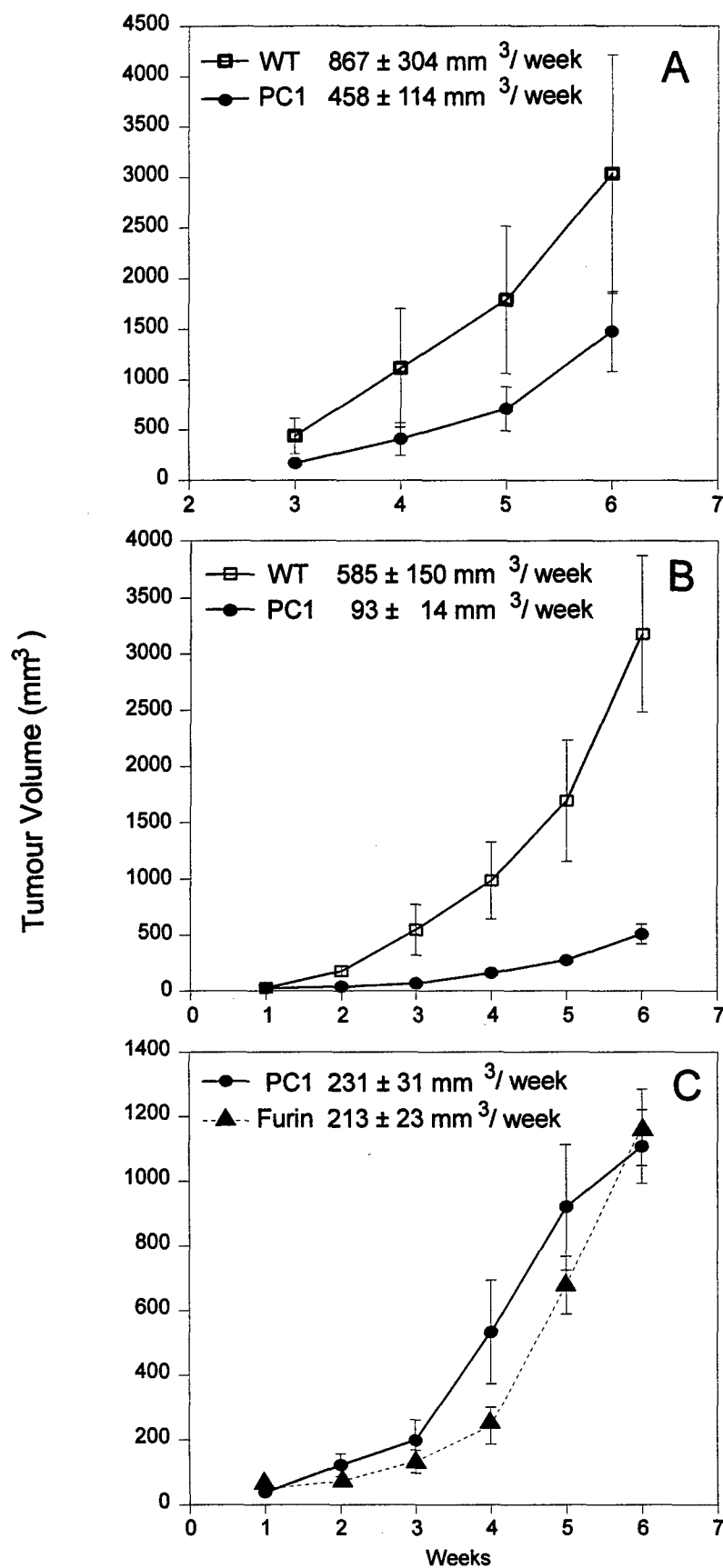
Numbers are mean ± S.E.M. (3 experiments)

\* denotes significant difference from clone WT;  $p < 0.05$

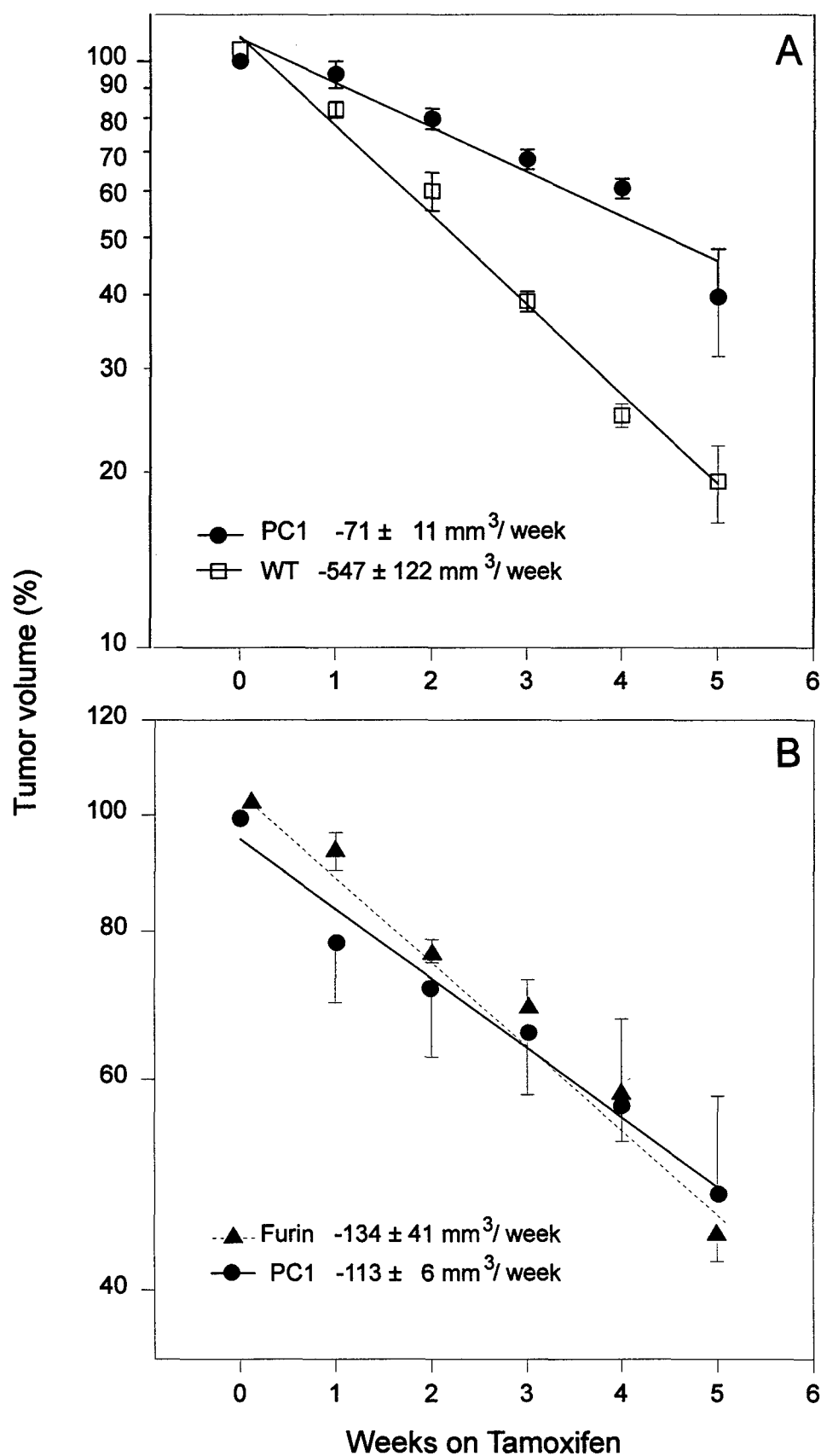














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